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Prevalence and transmission potential of Borrelia burgdorferi in three species of wildcaught Plestiodon spp. skinks of the southeastern United States

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To the Graduate Council:

I am submitting herewith a thesis written by Teresa Dianne Moody entitled "Prevalence and transmission potential of Borrelia burgdorferi in three species of wildcaught Plestiodon spp. skinks of the southeastern United States." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Wildlife and Fisheries Science.

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Prevalence and transmission potential of *Borrelia burgdorferi* in three species of wild-caught *Plestiodon* spp. skinks of the southeastern United States

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ABSTRACT

In the southeastern United States, blue-tailed skinks (*Plestiodon spp.*) are important hosts for *Ixodes scapularis* ticks, the principal vector of Lyme disease (LD) in this region. Skinks and other southeastern lizards are not thought to be reservoir competent for *Borrelia burgdorferi* sensu stricto (*Bbss*), the etiological agent of LD in the United States. . Lizard-feeding by southeastern *I. scapularis* may tend to suppress sylvatic cycles of *B. burgdorferi*, and thus may be an important reason why LD case rates in the Southeast are much lower than in the Northeast and upper Midwest. Nevertheless, some skinks in Florida and South Carolina have tested positive for *Borrelia* spp. bacteria. The aims of this project therefore were the following: i) to determine the natural prevalence of *Bbss* in *Plestiodon* spp. skinks from the Southeast; and ii) to determine whether or not skinks experimentally infested with *Bbss*-infected *I. scapularis* would become a source of *Bbss* infection for naive ticks. Forty skinks were caught in southeastern states, of which two (5%) tested positive for a *Borrelia* species (not *Bbss*). In the laboratory, 25 uninfected skinks were infested with *Bbss*-infected nymph *I. scapularis*. *Bbss* infection in laboratory-infected nymphs declined from 72% before feeding to 7% after feeding on these skinks, suggesting this feeding had a strong zooprophylactic effect. Only one skink subsequently transmitted *Bbss* to a single xenodiagnostic larva, and that infection was transient. In contrast, all infected positive control mice transmitted infection to multiple larvae for the duration of the 6-week study. Skinks in the Southeast are probably not an ecologically-significant wildlife reservoir of *Bbss*, and are not contributing directly to the LD cycle. The prevalence of other *Borrelia* species in skinks, and the possibility that such

bacteria could be acquired and transmitted by human-biting ticks, remains an avenue for further study.

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CHAPTER 1 - INTRODUCTION

1.1 INTRODUCTION

In the southeastern United States, where their ranges overlap, blue-tailed skinks (*Plestiodon spp.*) are important hosts for blacklegged ticks (*Ixodes scapularis*), which are the principal vector of *Borrelia burgdorferi* sensu stricto (*Bbss*) (Stanek et al., 2012), the etiological agent of Lyme disease (LD) (Apperson, et al., 1995; Kierans et al., 1996). Several studies have indicated that lizards are not reservoir competent for *Bbss*. This has led to speculation that lizard-feeding by southeastern *I. scapularis* suppresses sylvatic cycles of *B. burgdorferi*. This may explain why LD case rates in the Southeast are much lower than case rates in the Northeast (Apperson et al., 1993). Nevertheless, skinks in Florida and South Carolina have been reported positive for *Borrelia* spp. bacteria, although it is uncertain whether or not these are strains that cause borreliosis in humans (Clark et al., 2005). The aims of this project were the following: i) to determine the natural prevalence of *Bbss* in *Plestiodon* spp. skinks from the Southeast; and ii) to determine whether skinks infested with *Bbss*-infected *I. scapularis* would transmit that infection to naive ticks.

1.2. BACKGROUND

1.2.1. Lyme disease in humans

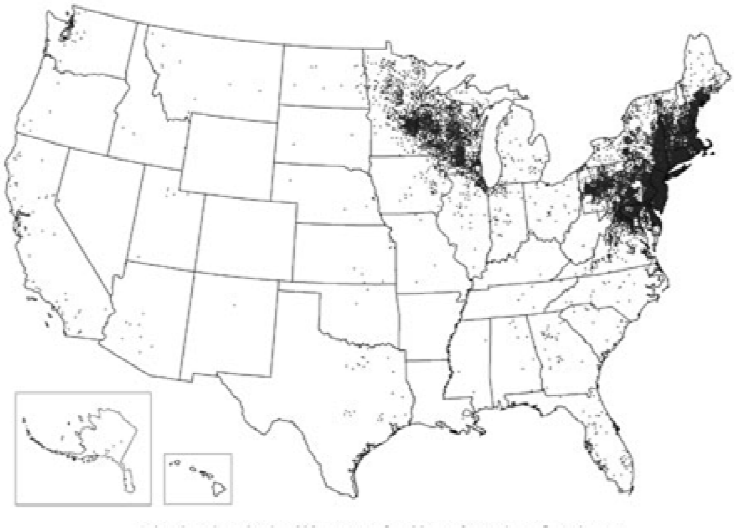
Lyme disease (LD) is the most commonly diagnosed vector-borne disease in the United States (CDC, 2012), with cases concentrated in the Northeast and northern Midwest (Figure 1). Several Lyme disease causing bacterial species are recognized in

Europe, including *Borrelia burgdorferi, B. afzelii, B. garinii, B. spielmanii* and *B. bavariensis* (Stanek et al., 2012). In the United States, *B. burgdorferi* sensu stricto (*Bbss*) is the only recognized LD pathogen (Stanek et al., 2012 for a review), although *B. bissettii* and *B. miyamotoi* have both recently been implicated in LD-like disease (Chowdri et al., 2013; Girard et al., 2011). Symptoms associated with LD in humans vary but can include a bull's-eye rash (erythema migrans), arthritic joints, and malaise (CDC, 2012; Stanek et al., 2012). Early-stage infection is usually readily treatable with doxycycline (Stanek et al., 2012). Disseminated infection is more problematic because the bacterium can evade the host immune system by down-regulating expression of surface proteins and lipoproteins (Cabello, 2007; Stanek et al., 2012). Left untreated, chronic LD can cause problems in patients for months or years. Furthermore, Post-Treatment Lyme Disease Syndrome (PTLDS) occurs in 10-20% of patients that were given the recommended antibiotic regimen (CDC, 2012; Stanek et al., 2012). Patients with PTLDS may take months to fully recover (CDC, 2012).

In 2011, there were 26,364 confirmed or probable LD case reports in the U.S. (CDC, 2012). Human LD confirmed cases are concentrated in the Northeast and northern Midwest (Figure 1.1). Southeastern confirmed cases of LD are much less common; 96% of the confirmed cases from 2011 were from 13 Northeastern and Midwestern states (CDC, 2012).

In contrast to the northern distribution of LD, the range of *I. scapularis* includes the entire coastal southeast (Figure 1.2). This mismatch of the distributions of vector ticks and human disease has led to ongoing speculation about why there are many vector ticks in the Southeast, yet few confirmed LD cases in the same areas. One hypothesis is that

low LD prevalence in the Southeast is associated with a latitudinal change in the key wildlife host utilized by the immature life-stages of these ticks. This host-shift hypothesis provided the motivation for this research project.



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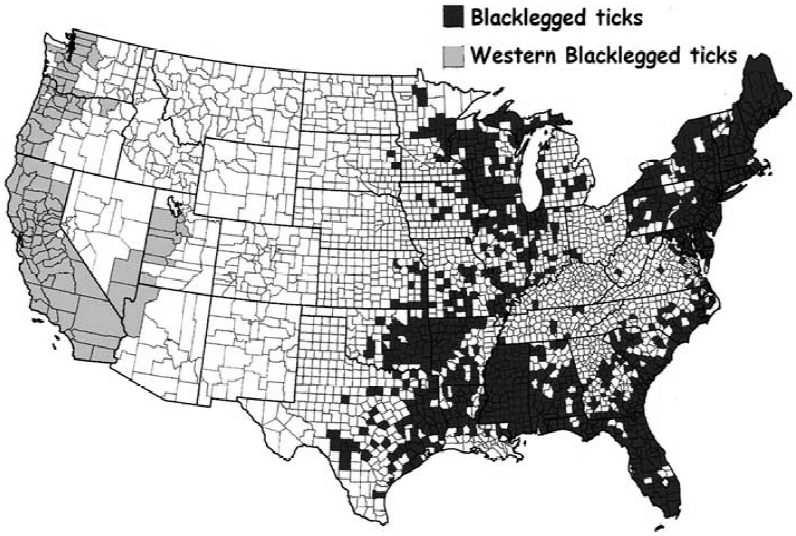

Figure 1.2. Distribution by county of *Ixodes scapularis* and *I. pacificus* in the United States as of 1998 (Dennis et al., 1998). These tick species are the key vectors of Lyme disease in the eastern and western U.S., respectively.

1.2.2. Sylvatic cycles of *Borrelia burgdorferi* in the Northeast and Midwest

The ecology of the vector tick (*I. scapularis* in the eastern U.S.; Kierans, et al., 1996) determines the cycle of infection and abundance of *Borrelia spp*. in the natural ecosystems. This tick is the most common vector for the LD pathogen in the eastern United States (Stanek et al., 2012). It utilizes three hosts during its life cycle; i.e., during each of its larval, nymph and adult stages (Stanek et al., 2012). The chance of larvae being infected from their parent (i.e., transovarial transmission) is extremely low or nil (Piesman et al., 1986; Stanek et al., 2012), so larvae do not transmit the pathogen to their hosts. Larvae obtain *Bbss* by feeding on an infected host, and thereafter typically remain infected into their nymph and adult stages (i.e., transstadial transmission). Consequently, nymphs and adults are the two life stages that can pass *Bbss* to susceptible hosts. Different hosts maintain infection for differing lengths of time. Mice (*Peromyscus spp.*) are the most abundant reservoir-competent hosts for *Bbss* in the northern ecosystems, although numerous other reservoir-competent birds and mammals also contribute to sylvatic *Bbss* cycles (Ostfeld and Keesing, 2000). An infected white-footed mouse can transmit the bacterium to a high (~92%) of the ticks that feed on it (LoGiudice et al., 2003). Whereas, chipmunks infected with *Bbss* were 75% infected, and meadow voles were 5.5% infected in a study conducted in Massachusetts (Mather et al., 1989).

1.2.3. Sylvatic cycles of *Borrelia burgdorferi* in the Southeast

The southeastern *Bbss* transmission cycle differs greatly from the Northeast and Midwest. Lyme Disease cases are infrequent; in Tennessee, for example, there were only

five confirmed LD cases in a population of over 6 million people in 2011 (CDC, 2012). Vector-competent *I. scapularis* occurs throughout the state, yet testing of >1000 ticks produced no detectable prevalence of *Bbss* in these populations from 2007-2008 (Rosen et al., 2009). Numerous strains and species of non-*Bbss Borrelia* have been found in southeastern wildlife and their associated ticks (Rudenko et al., 2009), but these appear to be maintained almost entirely by cryptic cycles not involving human-biting ticks.

Low LD incidence in the Southeast may arise because juvenile *I. scapularis* ticks in that region feed primarily on reservoir-incompetent lizards rather than on reservoir-competent rodents (e.g. Apperson et al., 1993; Ostfeld and Keesing, 2000). In a correlational analysis, Ostfeld and Keesing (2000) demonstrated that regions of the eastern U.S. with the highest lizard species richness had the least LD cases.

There are a few physiological studies supporting the hypothesis that lizards can be *Bbss* reservoir competent. Species such as the Western Fence Lizard (*Sceloporus occidentalis*) are incapable of becoming infected due to the presence of a complement lysing protein in the lizards' blood (Lane et al., 2006; Kuo et al., 2000). Nevertheless, *Borrelia spp.* competent lizards in Europe, such as *Lacerta spp.,* (Foldvari et al., 2009; Vaclav et al., 2011) and *Podarcis spp.* (Ragagli et al., 2010), have been reported. *Borrelia spp.* are also found in lizards in the southeastern U.S. (Clark et al., 2005; Levin et al., 1996). However, the reservoir and transmission capabilities of these lizards are uncertain.

1.2.4. Southeastern lizards and ticks

The Southeast has very different wildlife communities than the Northeast and Midwest, with a major difference being the abundance of reptiles. Reptiles, lizards

especially, are frequently more abundant than rodents in southeastern forests and use similar habitats (Apperson et al., 1993; Ostfeld and Keesing, 2000). Skinks are extremely common in many areas of the Southeastern United States. The "blue-tailed" skinks (*Plestiodon spp.)* are arguably the most common lizards seen in the woods as well as around residential areas (Conant and Collins, 1998). There are several species that comprise this "blue-tailed" skink group, including the common five-lined skink (*P. fasciatus*), the southeastern five-lined skink (*P. inexpectatus*), and the broad-headed skink (*P. laticeps*).

The only species of tick collected from lizards in recent eastern United States lizard tick studies were *I. scapularis* (e.g., Apperson et al., 1993; Clark et al., 2005; Giery and Ostfeld, 2007; Kollars et al., 1999; Levine et al., 1997; Swanson and Norris, 2007). Apperson et al. (1993) found up to 88% of *P. laticeps* in the southeast were infested with *I. scapularis*; whereas, Levine et al. (1997) reported that 13.8% of *P. inexpectatus*, 3% of *P. fasciatus*, and 7.4% of *P. laticeps* were infested. Similarly, Durden et al. (2002) found that 93% of the *P. laticeps* had attached larvae and 89% had nymphs and 80% of *P. inexpectatus* had larvae and 88% had nymphs. Therefore, it is apparent lizards are important blood meals for *I. scapularis* in the Eastern United States. Not only do *I. scapularis* juveniles feed on lizards, there have been documented loads of immature black-legged ticks as high as 394 ticks per *P. laticeps* lizard have been reported (Kierans et al., 1996). Even though these lizards can host many ticks, it is still unclear if they can act as reservoir hosts for *Bbss* acquired from ticks that feed on them.

1.3. RESEARCH QUESTIONS

The aim of this study was to better understand the potential role of *Plestiodon* skinks as reservoir hosts for *Bbss*. The natural prevalence of *Bbss* in wild-caught skinks was assessed by determining whether or not they would transmit *Bbss* infection to naïve, xenodiagnostic tick larvae acquired naturally or artificially. Naive lizards were experimentally infected with northern-strain *Bbss* to determine reservoir competency and bacterial persistence,

My research questions were as follows:

1. Are wild-caught skinks from southeastern states infected with naturally-occurring *Borrelia spp.?*

   And if so:

   1a) Are these *Borrelia* strains genetically similar to, or very different from, those found in northern enzootic cycles?

2. Will skinks experimentally infested with *Bbss*-infected *I. scapularis* become a source of infection to naive ticks?

   And if so:

   2a) How long will such lizards continue to act as a reservoir of *Bbss*?

Chapter 2 addresses the first of these questions and Chapter 3 addresses the second; both chapters are written in manuscript format to facilitate subsequent publication. Chapter 4 briefly summarizes my overall conclusions and discusses potential directions for future research on this topic.

CHAPTER 2 - *BORRELIA BURGDORFERI* PREVALENCE IN *PLESTIODON* SPP. SKINKS OF THE SOUTHEASTERN UNITED STATES

2.1 INTRODUCTION

Lyme disease (LD) is the most commonly diagnosed vector borne disease in the United States (CDC, 2012). In the US, *Borrelia burgdorferi* sensu stricto (*Bbss*) is the only recognized LD pathogen (Stanek et al., 2012 for a review), although *B. bissettii* and *B. miyamotoi* have both recently been implicated in LD-like disease (Chowdri et al., 2013; Girard et al., 2011).

In 2011, there were 26,364 confirmed or probable LD case reports in the U.S. (CDC, 2012). Confirmed cases are concentrated in the Northeast and northern Midwest (Figure 1.1). Southeastern confirmed cases of LD are much less common; 96% of the confirmed cases from 2011 were from 13 Northeastern and Midwestern states (CDC, 2012).

In contrast to the northern distribution of LD, the range of *I. scapularis* includes the entire coastal Southeast (Figure 1.2). This mismatch of the distributions of vector ticks and human disease has led to ongoing speculation about why there are many vector ticks in the Southeast, yet few confirmed LD cases in the same areas. One hypothesis is that low LD prevalence in the Southeast is associated with a latitudinal change in the key wildlife host utilized by the immature life-stages of these ticks. This host-shift hypothesis provided the motivation for this research project.

2.1.1. Sylvatic cycles of *Borrelia burgdorferi* in the Northeast and Midwest

The ecology of the vector tick (*I. scapularis* in the eastern U.S.; Kierans et al., 1996) determines the cycle of infection and abundance of *Bbss* in natural ecosystems. This tick is the most common vector for the LD pathogen in the eastern United States (Stanek et al., 2012). It utilizes three hosts during its life cycle; i.e., during each of its larval, nymph and adult stages (Stanek et al., 2012). The chance of larvae being infected from their parent (i.e., transovarial transmission) is nonexistant (Piesman et al., 1986; Stanek et al., 2012), so larvae do not transmit the pathogen to their hosts. Larvae typically obtain *Bbss* by feeding on an infected host, and thereafter typically remain infected into their nymph and adult stages (i.e., transstadial transmission). Consequently, nymphs and adults are the two life stages that pass *Bbss* to susceptible hosts. Mice (*Peromyscus spp.*) are the most abundant reservoir-competent hosts for *Bbss* in the northern ecosystems, although numerous other reservoir-competent birds, lizards and mammals contribute to sylvatic *Bbss* cycles (Ostfeld and Keesing, 2000). An infected white-footed mouse can transmit the bacterium to 40-90% of the ticks that feed on it (LoGiudice et al., 2003).

2.1.2. Sylvatic cycles of *Borrelia burgdorferi* in the Southeast

The southeastern *Bbss* transmission cycle differs greatly from the Northeast and Midwest. Lyme disease cases are infrequent; in Tennessee, for example, there were only five confirmed cases in a population of over 6 million people in 2011 (CDC, 2012). Vector-competent *I. scapularis* occur throughout the state, yet testing of >1000 ticks in 2007-2008 produced no detectable prevalence of *Bbss* in these populations (Rosen et al., 2009). Numerous strains and species of non-*Bbss Borrelia* have been found in

southeastern wildlife and their associated ticks (Rudenko et al., 2009), but these appear to be maintained almost entirely by cryptic cycles not involving human-biting ticks (Stromdahl and Hickling, 2012).

2.1.3. Southeastern lizards and ticks

The southeastern United States has different wildlife communities than the Northeast and Midwest, with a major difference being the abundance of reptiles. Reptiles, lizards especially, are frequently more abundant than rodents in southeastern forests and use similar habitats (Apperson et al., 1993, Ostfeld and Keesing, 2000). Skinks are extremely common in many areas of the Southeast. The "blue-tailed" skinks (*Plestiodon spp.)* are arguably the most common lizards seen in the woods, as well as around residential areas (Conant and Collins, 1998). There are several species that comprise this "blue-tailed" skink group, including the common five-lined skink (*P. fasciatus*), the southeastern five-lined skink (*P. inexpectatus*), and the broad-headed skink (*P. laticeps*).

Apperson et al. (1993) found up to 88% of broad-headed skinks (*P. laticeps*) in the southeast were infested with *I. scapularis*; whereas, Levine et al. (1997) reported that 13.8% of *P. inexpectatus*, 3% of *P. fasciatus*, and 7.4% of *P. laticeps* were infested. Similarly, Durden et al. (2002) found that 93% of the *P. laticeps* had larvae and 89% had nymphs and 80% of *P. inexpectatus* had larvae and 88% had nymphs. The only species of tick collected from lizards in recent eastern United States lizard tick studies were *I. scapularis* (e.g., Apperson et al., 1993; Clark et al., 2005; Giery and Ostfeld, 2007; Kollars et al., 1999; Levine et al., 1997; Swanson and Norris, 2007). Therefore, it is apparent that lizards are important blood meals for *I. scapularis* in the Eastern United

States. Not only do *I. scapularis* juveniles feed on lizards, there have been documented loads of immature black-legged ticks as high as 394 ticks on a single *P. laticeps* (Kierans et al., 1996). Even though these lizards can host many ticks, it is still unclear if they can act as reservoir hosts for *Bbss* acquired from ticks that feed on them.

In this Chapter, I aimed to assess the natural prevalence of *Borrelia* spp. in free-ranging skinks at several collection sites in the Southeast. The findings are discussed in relation to opposing views of other recent research studies regarding the importance of lizards for *Bbss* transmission in this region.

2.2 METHODS

Skink collection

From May through June 2012, skinks were captured alive at field sites in Tennessee, Georgia, Alabama, North Carolina and Florida (Table 2.1). Skinks were caught using a variety of methods, including hand capture, noosing, cricket luring, burlap traps, cover boards and pitfall/drift-fence arrays. To collect skinks using nooses, I made a slipknot out of waxed dental floss, tied it to the end of a fishing pole, slipped the open knot over the skink's head and swung the skink into a bucket. Cricket luring was done by skewering a cricket with a twig a little longer than its body, tying the cricket to the stick with waxed dental floss and then attaching the loose end of the lure to the end of a fishing pole. The cricket was then dangled in front of the targeted wild skink. When the skink grasped the cricket, it was swung into a bucket. Burlap traps consisted of a 1m x1m burlap sheet tied to the side of a tree (adapted from methods used by Mulder, 2012). Skinks that were attracted to the resulting shelter/insect fauna were captured by hand or

with a net. Cover boards consisted of plywood or tin roofing measuring 1m x1m placed on the ground to act as refugia for skinks. Cover boards were lifted periodically and skinks underneath were captured by hand. Pitfall/drift-fence arrays consisted of a set of four 20 L buckets buried into the ground in a cross shape with one in the middle, spaced 10m apart, and connected by 61 cm high aluminum flashing buried about 15 cm into the ground. Between fieldtrips, a lid was placed on each bucket to prevent non-target captures.

Table 2.1. Capture sites and habitat types sampled for skinks in 2012, ordered by decreasing latitude. Coordinates were determined using Google Earth satellite photographs.

State	**Site** name	**Habitat**	**Latitude**	**Longitude**
TN	OAKR	Hardwood forest	36.03	-84.20
TN	UTK	Anthropogenic	35.94	-83.94
NC	MNWR	Pocosin swamp	35.47	-76.32
TN	AEDC	Hardwood forest	35.30	-86.10
GA	Wildwood	Anthropogenic	34.90	-85.48
AL	OTNF	Coniferous forest	32.95	-87.46
FL	TTRS	Pine/hardwood forest	30.66	-84.21

Upon capture, I assessed each skink's species identity, age (juvenile or adult), and gender, and collected data on weight (nearest 0.5g), snout-vent-length, head width and tail length (all nearest 1mm). Species identity, age (juvenile or adult) and gender were

determined from morphological features with the aid of a field guide (Conant and Collins, 1991).

Assessment of tick infestation and *Borrelia* spp. infection

Captured skinks were transferred to individual cages at the University of Tennessee Institute of Agriculture (UTIA). Their naturally acquired ticks (i.e., larvae and nymphs attached to the skinks at time of capture) were allowed to feed to repletion. As the engorged ticks detached, they were collected and stored in 70% EtOH for later PCR testing.

The *Bbss* infection status of each skink was assessed by testing larvae that had engorged and detached from the skinks. This 'larval xenodiagnosis' procedure is a more sensitive test for *Bbss* than is direct testing of the skink's blood or tissue (J. Tsao, pers. comm.). The engorged nymphs also present on some skinks were not used in the assessment because of the possibility that they had become infected with *Borrelia spp.* from their earlier larval blood meal on a different host.

To ensure an adequate sample of larvae to assess the *Bbss* infection status of each skink, naturally-attached larvae were supplemented by applying up to 50 laboratory-raised larvae to each skink soon after capture, if the skink had less than five naturally acquired larvae. The procedure for applying and collecting these additional larvae is described in Chapter 3.

The DNA of engorged larvae were extracted in pools of up to 10 larvae per extraction, with a minimum of three extractions, using Qiagen Blood and Tissue Extraction kits. I then tested for the presence of *Borrelia* spp. using Real-time PCR

targeting the 23S rRNA gene as described by Courtney et al. (2004). All samples which resulted in a reaction above the critical threshold were considered a positive result.

Prior to employing the real-time PCR system on my samples, I evaluated the possibility that skink blood within engorged ticks could cause inhibition of the qPCR to recognize the *Borrelia* in the samples. I combined extracted DNA samples from nymphs that fed on two negative mice with two positive skinks in two individual tests, and two positive mice with two positive skinks in two individual tests. Each sample had DNA concentrations less than 100 ng/µL. I ran qPCR tests on the combined DNA from ticks that fed on skinks and mice a dilution series as follows: 2 µL mouse fed tick DNA, 1.5 µL of mouse fed tick DNA: 0.5 µL of skink fed tick DNA, 1 µL of mouse fed tick DNA: 1 µL of skink fed tick DNA, and vice versa. Each dilution produced a positive qPCR reaction, suggesting no inhibition.

To determine the species identity of any *Borrelia* detected, DNA from larval pools that tested positive by real-time PCR was evaluated using a nested PCR targeting the intergenic spacing region between the single 16S rRNA and the first of two 23S rRNA (Bunikis et al., 2004). PCR product was run on an ethidium bromide gel (400 mL) at 50V for up to 8 hours to make bands apparent. Bands in the 900-1500 base pair region of the resulting gel were removed, cleaned with a Zymo Clean Gel DNA Recovery Kit (manufacturer and address), and sent for sequencing at the UT Sequencing Laboratory. The minimum concentration of DNA needed for sequencing was 10 ng/ 100 bases.

Nested IGS PCR products that could not be successfully sequenced and aligned with a known sequence at UT were sent to the Stephenson laboratory at the University of Kentucky where the Stevenson team re-amplified the product with the same nested IGS

primers. They then TA-cloned the PCR products using an Invitrogen PCR 2.1 plasmid kit for direct cloning of PCR amplicons. Subsequently, they sequenced inserts with primers in the vector (oligos M13 forward & reverse) and compared the result to previously published sequences using NCBI BLAST.

Persistence of natural infection

To investigate the persistence of any natural infection, suspect-positive skinks were re-infested with 30-50 xenodiagnostic larvae seven weeks post capture. The DNA of these larvae was extracted and tested with qPCR using the procedures described above.

2.3 RESULTS

Tick infestation

Forty skinks were collected between March and June 2012 at the seven sites listed in Table 2.1. This total comprised 15 *Plestiodon fasciatus* (PLFA; 6 adult and 9 juvenile), 6 *Plestiodon inexpectatus* (PLIN; 2 adult and 4 juvenile), and 19 *Plestiodon laticeps* (PLLA; 11 adult and 8 juvenile). The captured skinks originated from Tennessee (15), Georgia (1), Alabama (3), North Carolina (4) and Florida (17). Capture data for each individual skink are summarized in Table 2.2.

The majority of the skinks (29 of 40; 73%) were infested with immature *I. scapularis*. *P. laticeps* were 89% infected; whereas *P. inexpectatus* and *P. laticeps* were 67% and 56% infected, respectively. These differences were not statistically significant (Chi-square Test of Association; $X^2 = 4.65$, 2df, P = 0.10).

A total of 554 naturally-acquired *I. scapularis* ticks (499 larvae and 55 nymphs) were collected from the skinks. The median number of nymphs per skinks was 0 (range 0 – 17), and the median number of larvae was 12.5 (range 0 – 87). Nymph loads were highest in Florida in June. Larval loads were highest in Florida in April. Median numbers of larvae on PLLA, PLIN and PLFA were 27, 0 and 0, respectively. Median numbers of nymphs on PLLA, PLIN and PLFA were 2, 0 and 0, respectively.

Borrelia spp. prevalence

Based on qPCR of DNA from the pooled xenodiagnostic larvae, the prevalence of *Borrelia* spp. in these wild skinks was 5% (2 of 40). Both infected individuals (Skinks 21 and 28) were *P. laticeps* collected from Tall Timbers Research Station, near Tallahassee, FL. Skink 21 had two natural larvae pools test positive, each of these pools contained 10 larvae. Skink 28 had one natural larvae pool test positive, this pool contained ~9 larvae.

Nested 16S-23S PCR of the larval DNA resulted in strong bands from several larval pools from both of these skinks (Plate 1). Bands were in the 1000-1500bp range, and so were inconsistent with *Bbss* infection (expected bands <1000bp). An attempt to sequence the DNA from these bands at UT was unsuccessful; the sequences that resulted were not the full length of the targeted base pairs and so the sequences would not align.

The Stevenson laboratory undertook further analysis of PCR product from the three larval pools that had produced the strongest bands in the nested PCR analysis (one pool from skink 21 and two from skink 28). DNA clones originating from both of the larval samples from skink 28 were sequenced successfully. Both sequences appeared to

be from the same *Borrelia* species; however no close match was obtained to any *Borrelia* species in the NCBI database.

Table 2.2. Species identity, capture date and location for each skink, plus age and gender (J = juvenile, A = adult), weight and other morphometric data (S-V = snout-vent length, Head = head width, Tail = tail length), and the number larvae (LL) and Nymphs (NN) attached at time of capture. PLFA = *Plestiodon fasciatus*; PLLA = *P. laticeps*; PLIN= *P. inexpectatus.*

Skink #	Species	Capture date	Capture site	Age	Gender	Weight (g)	S-V (mm)	Head (mm)	Tail (mm)	LL	NN
1	PLFA	3/23/2012	Wildwood	J	?	2.0	42		21	0	0
2	PLFA	3/24/2012	AEDC	A	M	6.0	58	10	75	0	1
3	PLIN	3/31/2012	OTNF	A	F	8.0	66	10	64	0	2
4	PLIN	3/31/2012	OTNF	J	?	2.0	45	8	68	0	0
5	PLFA	4/15/2012	AEDC	A	?	5.0	60	10	35	0	1
6	PLIN	4/12/2012	MNWR	A	F	7.0	68	10	18	0	0
7	PLFA	4/13/2012	MNWR	A	M	4.0	56	10	68	0	0
8	PLLA	4/24/2012	TTRS	A	M	30.0	91	23	155	40	3
9	PLLA	4/24/2012	TTRS	A	M	28.0	97	22	145	27	1
10	PLLA	4/24/2012	TTRS	A	M	24.0	90	20	157	39	2
11	PLLA	4/24/2012	TTRS	A	F	40.0	109	12	135	27	0
12	PLLA	4/24/2012	TTRS	A	M	44.0	113	23	115	37	0
13	PLLA	4/24/2012	TTRS	A	F	25.0	96	16	103	12	0
14	PLLA	4/24/2012	TTRS	A	M	44.0	111	29	123	87	4

Table 2.2 continued

15	PLFA	5/6/2012	AEDC	A	M	7.5	61	10	80	0	0
16	PLLA	5/6/2012	AEDC	A	M	31.5	102	25	102	0	0
17	PLFA	5/16/2012	Oak Ridge	A	M	6.5	68	11	91	0	0
18	PLFA	5/27/2012	Oak Ridge	J	?	4.0	52	10	74	0	0
19	PLFA	6/5/2012	Oak Ridge	A	M	7.5	67	13	59	1	1
20	PLFA	6/5/2012	Oak Ridge	J	?	7.0	69	14	90	1	0
21	PLLA	6/14/2012	TTRS	A	M	41.0	120	25	95	39	17
22	PLLA	6/14/2012	TTRS	A	M?	33.0	101	20	159	22	3
23	PLFA	6/14/2012	TTRS	J	?	8.5	65	12	80	2	0
24	PLLA	6/14/2012	TTRS	J	M?	34.0	105	21	134	46	1
25	PLLA	6/14/2012	TTRS	J	?	8.5	81	15	137	2	3
26	PLLA	6/14/2012	TTRS	J	?	21.5	88	14	131	7	6
27	PLLA	6/14/2012	TTRS	J	?	32.5	108	19	165	20	7
28	PLLA	6/14/2012	TTRS	A	M	13.5	82	12	129	12	1
29	PLLA	6/14/2012	TTRS	J	?	15.0	87	19	136	0	0
30	PLLA	6/14/2012	TTRS	J	?	17.0	81	16	108	9	0
31	PLIN	6/14/2012	MNWR	J	?	8.5	65	11	95	4	0
32	PLFA	6/14/2012	MNWR	J	?	11.5	75	12	131	2	1
33	PLIN	6/14/2012	OTNF	J	?	3.5	54	9	80	2	0
34	PLFA	6/19/2012	Oak Ridge	J	?	3.5	53	11	-	0	0
35	PLFA	6/19/2012	Oak Ridge	J	?	5.0	65	8	62	3	0
36	PLLA	6/19/2012	Oak Ridge	J	?	4.5	52	9	71	2	0
37	PLIN	6/23/2012	Oak Ridge	J	?	4.0	59	8	18	33	1
38	PLFA	6/23/2012	Oak Ridge	J	?	3.5	50	9	77	12	0
39	PLLA	6/23/2012	Oak Ridge	J	?	2.5	44	8	62	11	0
40	PLFA	6/27/2012	UTK	J	?	3.5	52	7	110	0	0

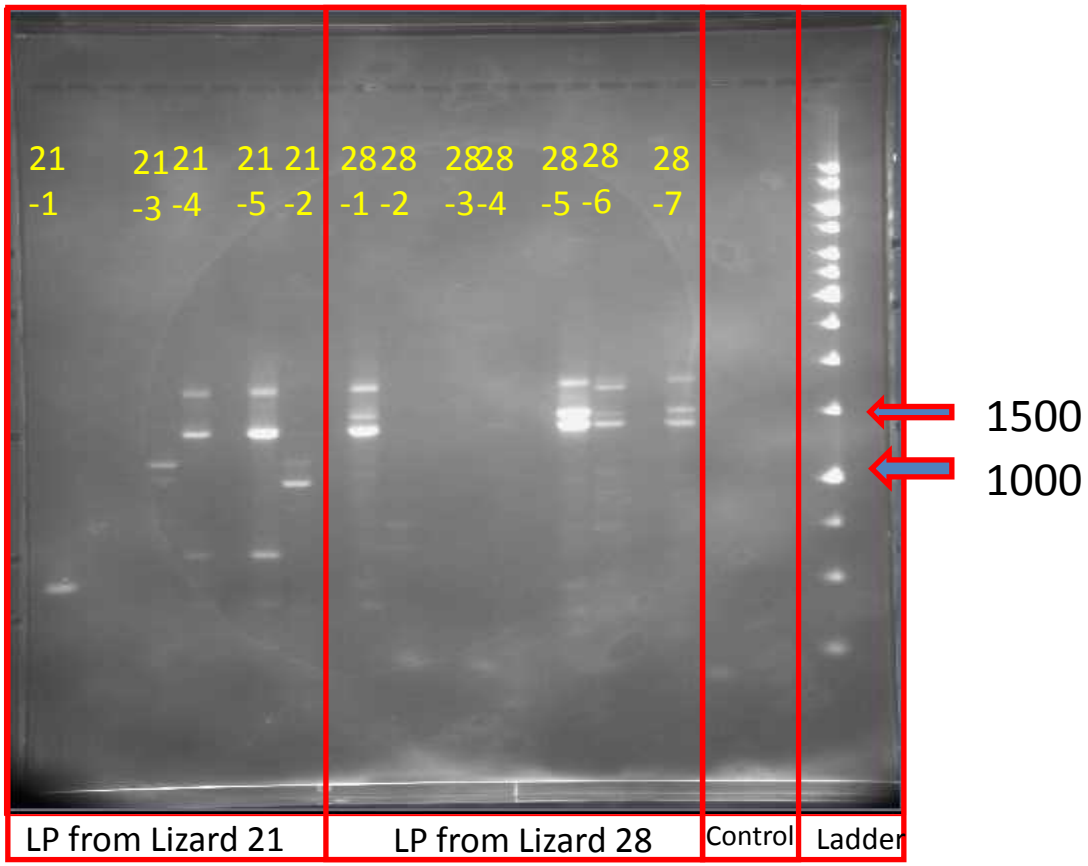

Plate 1. Gel electrophoresis image of nested PCR product of the 16S-23S intergenic spacer (IGS) of *Borrelia burgdorferi* sensu lato from xenodiagnostic *Ixodes scapularis* larval pools (LPs) that fed on two *Borrelia*-positive skinks (N=5 pools for skink 21; N=7 pools for skink 28). The negative control consisted of water. Thermo Scientific Gene Ruler™ 1 kb DNA ladder was used as the molecular weight ladder.

Persistence of *Borrelia* infection

Additional xenodiagnostic larvae were allowed to feed on skinks 21 and 28 seven to eight weeks after capture to assess the persistence of the *Borrelia* infection. None of these xenodiagnostic larvae tested positive for *Borrelia* spp. (Table 2.3).

Table 2.3. Timeline of the persistence trial conducted on two *Borrelia*-positive *Plestiodon laticeps* collected in Florida in April 2012. Naturally attached larvae were tested in pools of up to 10 larvae per sample, with at least three extractions tested per skink. Xenodiagnostic larvae were applied 47 and 55 days after the date of capture.

		Skink #21	**Skink #28**
Date of capture	6/14/2012		
Natural larvae collected into EtOH		39	12
- Larval pools testing positive		2 of 3	1 of 3
Natural nymphs collected into EtOH		9	2
- Nymphs testing positive		1 of 9	0 of 2
Xenodiagnostic larvae applied	7/31/2012	50	35
- Engorged larvae into EtOH		6	4
- Larval pools testing positive		0 of 3	0 of 3
Xenodiagnostic larvae applied	8/8/2012	50	50
- Engorged larvae into EtOH		5	6
- Larval pools testing positive		0 of 3	0 of 3

2.4 DISCUSSION

Lyme disease is a very important human disease that causes a great deal of human illness in the North and Midwest, but it has been a topic of controversy in the South. My study supports the hypothesis that skinks are suppressing the cycle of *Bbss*.

Skinks have been thought to be dilution hosts due to their potential reservoir incompetency (Apperson et al., 1993). Ogden and Tsao (2009) argue that the presence of an incompetent reservoir alone will not dilute the prevalence of *Bbss* in ticks. However, it appears the *I. scapularis* juvenile ticks may prefer lizards and are the only ticks found on lizards in the Southeast (Apperson et al., 1993; Kerr, 2012). Therefore, if juvenile *I. scapularis* are preferentially selecting less competent reservoirs, such as *Plestiodon* skinks, then the ticks are not feeding on the better LD reservoirs. Since ticks feed on skinks in high quantities, they may be increasing the amount of *I. scapularis* adults predating humans. Therefore, lizards may be assisting with the dilution of *Bbss* and *Bbsl* (Rudenko, 2009) in the Southeast, while increasing *I. scapularis* populations. Not only may they be diluting the pathogen, but their behavior may be decreasing human exposure to potentially infected *I. scapularis* nymphs. If *I. scapularis* nymphs are behaviorally selecting areas where *Plestiodon* skinks reside and the skinks are diurnal, like humans, then the skinks may be diverting those ticks (Apperson et al., 1993).

There are possibly reservoir competent lizards in the Southeast, since reservoir competent lizards have been documented in Europe. Reservoir competent European lizards include *Lacerta spp.* (Foldvari et al., 2009; Vaclav et al., 2011) and *Podarcis spp.* (Ragagli et al., 2010). Also, there are several studies reporting *Borrelia spp.* found in lizards in the Southeast and Europe (Clark et al., 2005; Levin et al., 1996). The evidence

of *Borrelia spp.* in two wild skinks (5%, n=40) supports Clark et al. (2005) reports, but I did not find any known species of *Borrelia* in the skinks. Clark et al. (2005) reports of up to 58% *Bbsl* infection in wild *P. laticeps* and 65% infection in wild *P. fasciatus* when tested with the *flaB* PCR assay. The variance of their results from mine may be due to the specificity of my testing methods. PCR methods of Courtney (2004) detected *Bbss* and *Bbsl* species and therefore may not be detecting low prevalence presence of other *Borrelia* species (2004). Although it was able to detect the potentially unknown *Borrelia spp.*, it does not seem to detect all known *Borrelia spp.* (14 spp., Rudenko et al., 2009). Further investigation is required to determine the prevalence of this potentially unknown *Borrelia* in natural skink populations.

Although some evidence of natural *Borrelia spp*. in wild lizards was found, most *Borrelia* spp. are not known to be pathogenic to humans (Stanek et al., 2012). More research needs to be conducted to determine the human pathogen potential of the *Borrelia* from the wild lizards. My results suggest lizards are not major reservoirs of *Bbss*. Even if infection can be obtained, my research supports the contention that lizards do not maintain infection. Future studies might focus on areas with confirmed cases of LD, high numbers of ticks and large populations of *Plestiodon* skinks may yield more of this unknown *Borrelia* sp., which would be useful for determining pathogen capabilities.

CHAPTER 3 – AN EXPERIMENTAL ASSESSMENT OF THE RESERVOIR COMPETENCY OF *PLESTIODON* SPP. SKINKS FOR *BORRELIA BURGDORFERI* SENSU STRICTO

3.1 INTRODUCTION

Lyme disease (LD) is the most commonly diagnosed vector borne disease in the United States (CDC, 2012), with cases concentrated in the Northeast and northern Midwest (Figure 1.1). Several Lyme disease causing bacterial species are recognized in Europe, including *Borrelia burgdorferi, B. afzelii, B. garinii, B. spielmanii* and *B. bavariensis* (Stanek et al., 2012). In the United States, *B. burgdorferi* sensu stricto (*Bbss*) is the only recognized LD pathogen (see Stanek et al., 2012 for a review), although *B. bissettii* and *B. miyamotoi* have both recently been implicated in LD-like disease (Chowdri et al., 2013; Girard et al., 2011). In 2011, there were 26,364 confirmed or probable LD case reports in the U.S.; 96% of those were from 13 northeastern and Midwestern states (CDC, 2012).

In contrast to the northern distribution of LD, the range of *I. scapularis* includes the entire coastal Southeast (Fig. 1.2). This mismatch of the distributions of vector ticks and human disease has led to ongoing speculation about why there are many vector ticks in the Southeast, yet few confirmed LD cases in the same areas. One hypothesis is that low LD prevalence in the Southeast is associated with a latitudinal change in the key wildlife host utilized by the immature life-stages of these ticks. This host-shift hypothesis provided the motivation for this research project.

3.1.2 Sylvatic cycles of *Borrelia burgdorferi* sensu stricto in the Northeast and Midwest

The ecology of the vector tick (*I. scapularis* in the eastern U.S.; Kierans et al., 1996) determines the cycle of infection and abundance of *Borrelia spp*. in the natural ecosystems. This tick is the most common vector for the LD pathogen in the eastern United States (Kierans, 1996; Stanek et al., 2012). It utilizes three hosts during its life cycle; i.e. during each of its larval, nymph and adult stages (Stanek et al., 2012). The chance of larvae being infected from their parent (i.e., transovarial transmission) is nonexistent (Piesman et al., 1986; Stanek et al., 2012), so larvae do not transmit the pathogen to their hosts. Larvae obtain *Bbss* by feeding on an infected host, and thereafter typically remain infected into their nymph and adult stages (i.e., transstadial transmission).

Consequently, nymphs and adults are the two life stages that can pass *Borrelia spp*. to susceptible hosts. Different hosts maintain infection for differing lengths of time. Mice (*Peromyscus spp.*) are the most abundant reservoir-competent hosts for *Bbss* in the northern ecosystems, although numerous other reservoir-competent birds and mammals also contribute to sylvatic *Bbss* cycles (Ostfeld and Keesing, 2000). An infected white-footed mouse can transmit the bacterium to a high (~92%) of the ticks that feed on it (LoGiudice et al., 2003). Chipmunks infected with *Bbss* were 75% infected, and meadow voles were 5.5% infected in a study conducted in Massachusetts (Mather et al., 1989).

3.1.3 Sylvatic cycles of *Borrelia burgdorferi* sensu stricto in the Southeast

The southeastern *Bbss* transmission cycle differs from the Northeast and Midwest. LD cases are infrequent; for example, in Tennessee, there were only five confirmed cases in a population of over 6 million people in 2011 (CDC, 2012). Vector-competent *I. scapularis* occur throughout the state, yet testing of >1000 ticks in 2007-2008 produced no detectable prevalence of *Bbss* in these populations (Rosen et al., 2009). Numerous strains and species of non-*Bbss Borrelia* have been found in southeastern wildlife and their associated ticks (Rudenko et al., 2009), but these appear to be maintained almost entirely by cryptic cycles not involving human-biting ticks.

Low LD incidence in the Southeast may arise because juvenile *I. scapularis* ticks in that region feed primarily on reservoir- incompetent lizards rather than on reservoir-competent rodents (e.g. Apperson et al., 1993, Ostfeld and Keesing, 2000). In a correlational analysis, Ostfeld and Keesing (2000) demonstrated that regions of the eastern U.S. with the highest lizard species richness had the least LD cases.

Several physiological studies support the hypothesis that most lizards are not *Bbss* reservoir competent. Species such as the Western Fence Lizard (*Sceloporus occidentalis*) are incapable of becoming infected due to the alternative complement pathway (Lane et al., 2006; Kuo et al., 2000). In contrast, however, several European lizards including *Lacerta spp.* (Foldvari et al., 2009; Vaclav et al., 2011) and *Podarcis spp.* (Ragagli et al., 2010) are reservoir competent for *Borrelia* species, and there are several studies reporting *Borrelia* spp. found in lizards in the southeastern U.S. (Clark et al., 2005; Levin et al., 1996).

3.1.4. Southeastern skinks and ticks

The Southeast has very different wildlife communities than the Northeast and Midwest, with a major difference being the abundance of reptiles. Reptiles, lizards especially, are frequently more abundant than rodents in southeastern forests and use similar habitats (Apperson et al., 1993, Ostfeld and Keesing, 2000). Skinks are extremely common in many areas of the Southeastern United States. The "blue-tailed" skinks (*Plestiodon spp.)* are arguably the most common lizards seen in the woods as well as around residential areas (Conant and Collins, 1998). There are three species that comprise this "blue-tailed" skink group and include the common five-lined skink (*P. fasciatus*), southeastern five-lined skink (*P. inexpectatus*), and the broad-headed skink (*P. laticeps*).

Several studies have reported *I. scapularis* loads on lizards in the Southeast. Apperson et al. (1993) found up to 88% of broad-headed skinks (*P. laticeps*) infested with *I. scapularis*. Levine et al. (1997) found 13.8% of *P. inexpectatus*, 3% of *P. fasciatus*, and 7.4% of *P. laticeps* infested. Durden et al. (2002) found out of the skinks they caught, 93% of the *P. laticeps* had larvae and 89% had nymphs and 80% of *P. inexpectatus* had larvae and 88% had nymphs. In these studies, all ticks found infesting lizards were immature *I. scapularis* (i.e., larvae or nymphs). Clark et al., (2005), Giery and Ostfeld (2007), Kollars et al. (1999) and Swanson and Norris (2007) similarly report only *I. scapularis* on skinks.

Black-legged tick infestations on reptiles are typically more prevalent than on rodents in southeastern habitats. For example, Apperson et al. (1993) found higher infestation on lizards (36.7%) than on rodents (17.8%) in North Carolina. Another study

that looked at relative abundance of *I. scapularis* on various hosts determined *P. laticeps* were 60% infested, *P. fasciatus* were 44% infested, and *P. inexpectatus* were 25% infested, whereas *Peromyscus* were only 6.5% infested (Kerr, 2012). Skinks can serve as a host to many immature ticks at once; *P. laticeps* can carry loads of immature black-legged ticks as high as 394 juvenile ticks per individual (Kierans, et al., 1996). It is clear, therefore, that skinks are important blood meals for *I. scapularis* in the Southeast. The high abundance of ticks on skinks versus rodents suggests these lizards play an important role in the ecology of LD and other pathogens vectored by *I. scapularis*.

In this Chapter, I present a laboratory experiment that investigates whether naturally-infected and laboratory-infected *I. scapularis* nymphs can pass *Bbss* infection to uninfected *Plestiodon* spp. skinks in such a way that those lizards would then be capable of transmitting that infection on to naïve, xenodiagnostic larvae. My findings are discussed in the context of the implications if northern *Borrelia*-infected *I. scapularis* populations were to expand into southeastern states in coming decades.

3.2 METHODS

Skink and mouse husbandry

As described in Chapter 2, wild skinks were collected from multiple field sites in Tennessee, Alabama, North Carolina, and Florida during 2012 (Table 2.1) and transferred to the Johnson Animal Research and Teaching Unit at the University of Tennessee (UT). Each skink was housed individually in a plastic tub ranging in volume from 0.5 L to 29 L, depending on the size of the skink. Skinks were fed a diet of protein- and calcium-rich live crickets dusted with reptile vitamins (Fluker's reptile vitamin with Beta Carotene;

http://www.flukerfarms.com/repta-vitamin.aspx), and provided water *ad libitum*. The tub substrate consisted of a paper towel dampened daily to increase and maintain a high humidity. The towels improved visibility of detached ticks and helped keep the cage clean. Room temperature was maintained at constant 82F on a 12h light: 12h dark cycle.

ICR-strain laboratory mice (*Mus musculus*) from Harlan laboratories (*www.harlan.com*) for use as experimental controls were maintained in the Cherokee Veterinary Building at UT at 70F on a 12h light: 12h dark cycle. Mice were provided with *ad libitum* water and commercial mouse pellets and were initially housed together in plastic tubs for a 1 week acclimatization period. During the experiment, they were held in individual metal cages with wire bottoms, suspended over water trays.

Only skinks uninfected with *Bbss* were used in the experimental infection trial. Infection status was assessed by PCR-testing each skink's naturally acquired larvae, plus up to 50 supplemental laboratory-applied larvae, as described in Chapter 2. Upon completion of the experiments, skinks that remained negative for *Borrelia* infection were transferred to a breeding colony at the Burke laboratory at Hofstra University. The mice were euthanized using 1.0-1.5 ml of sodium pentobarbital. All husbandry procedures were approved by UTIA's IACUC (Protocol #2010).

Xenodiagnosis

Larval *I. scapularis* were used to assess the skinks' initial infection status (Chapter 2) and also during the experimental trials. Xenodiagnostic larvae applied during the first experimental trial were obtained by collecting engorged female *I. scapularis* in fall 2011 from deer check stations in Tennessee and South Carolina and allowing them to oviposit at UT in a humidity chamber containing a $MgSO_4$ (14.4 M) solution that

maintained ~90% relative humidity (Stafford, 1994). Larvae applied in the second experimental trial were derived from engorged females obtained from the Tick Laboratory at the University of Oklahoma (http://www.cvm.okstate.edu/) and from batches of larvae supplied by CDC Atlanta, and maintained thereafter at UT in 90% humidity chambers. Given the absence of transovarial transmission of *B. burgdorferi* in *I. scapularis* (Piesman et al., 1986; Stanek et al., 2012), unfed larvae were assumed uninfected with *Bbss*.

To encourage attachment of xenodiagnostic larvae, each skink was briefly restrained in a narrow, open-ended bag constructed from food sealer plastic. The bags were 28cm in length, sealed to the width of the skink, and perforated for ventilation. Approximately 30-50 larvae were placed inside each bag. A skink was then added to the bag, which was then held closed using binder clips. Each skink was restrained in its bag for three hours to allow for larval attachment and then returned to its individual tub. All unattached larvae were counted to estimate the number of attached ticks. The skink bag remained open in the tub overnight to encourage further attachment by the remaining larvae, and then was removed. Mice were treated in a similar manner, with approximately 30-50 larvae were applied to each control mouse. Mice were sedated with ketamine/xylazine, dosed at 0.1 mL/10g with 80mg/kg ketamine (100mg/mL) and 6mg/kg xylazine (20 mg/mL). Ticks were then applied directly to each mouse's ears and fur using small paint brushes. Sedated mice were restrained inside paper towel tubes with the ends closed for three hours to allow for larval attachment and then released back into their individual cages.

Recovery of engorged larvae that had detached from the skinks was initially done by collecting the ticks from the damp paper towel substrate. Skinks sometimes consumed these ticks, so in subsequent trials, wire platforms (6 mm mesh) were constructed that raised the skinks 5cm above a layer of water added to the bottom of their tubs. Engorged ticks dropped through the mesh into water, which put them out of reach of the skink and protected them from desiccation. Mice were placed on mesh platforms over individual water pans. Personal protection equipment used during these procedures included Tyvek tops and booties as well as latex gloves. Biohazard stickers were placed on any cages where potentially infected ticks or hosts were housed.

Ticks that detached from skinks and mice were collected daily from the water with paint brushes and randomly assigned into one of two of the following groups: 1) larvae that were tested while still engorged, and 2) larvae that were allowed to molt into nymphs before testing. Engorged larvae were preserved for testing in individual host vials containing 70% ethanol. Larvae assigned to the molting group were washed for about 30-s in a bath of 10% liquid bleach and stored in a humidity chamber.

Experimental infection

To investigate whether ticks carrying *Bbss* were capable of infecting skinks, I undertook two experimental infection trials using the skinks that I had determined to be naturally uninfected through PCR-testing with the procedure described in Chapter 2.

Infection Trial 1: Naturally infected nymphs

Wild nymphs were collected by collaborators in LD endemic areas of Wisconsin and Rhode Island in spring 2012. Nymphs were shipped immediately to UT and housed

in humidity chambers (90% RH). *Bbss* infection prevalence among nymphs from these areas was anticipated to be to be ~30% (J. Tsao, pers. comm.); a subset of the nymphs was tested by real-time PCR targeting the 23S rRNA gene to determine their actual prevalence (using the protocol of Courtney et al., 2004).

In July 2012, a group of 11 uninfected skinks were each exposed to 20 of these infected wild nymphs using the restraint bags described above. Assuming 30% prevalence, feeding by five nymphs would represent a >80% chance of each skink being exposed to *B. burgdorferi*. The skinks were then held over water. Water baths were checked daily; detached nymphs were collected and tested by 23S real-time PCR to determine whether there had been any change in their infection prevalence as a consequence of having fed on the skinks.

At 3 weeks post-challenge, ~30 xenodiagnostic larvae were applied to each skink to determine its infection status. Larvae were allowed to feed until they detached. These post-exposure engorged xenodiagnostic larvae were pooled into three extraction groups per skink as follows: if more than 30 larvae were collected, 10 larvae were tested per pool; if less than 30 larvae, the total was divided into 3 equal-sized pools from each individual. Some skinks were capable of feeding few engorged larvae (<5); in these cases, I applied a second round of ~30 xenodiagnostic larvae approximately 10 days after the 3-week infestation to increase my sample size.

DNA was extracted from engorged larvae in pools of up to 10 larvae per extraction using Qiagen Blood and Tissue Extraction kits. The DNA samples were then tested for the presence of *Borrelia* spp. using the same 23S real-time PCR described previously for the engorged nymphs (Courtney, 2004).

Five ICR-strain mice were included in the trial as an experimental control. These were challenged with wild nymphs in the same way as the skinks (i.e., the same number of nymphs from the same locations as were used on the skinks). I tested engorged nymphs collected post-feeding on mice, and Week 3 xenodiagnostic larvae on mice in the same way as for the skinks.

Infection Trial 2: Laboratory-infected nymphs

In October 2012, a second group of 20 skinks were each exposed to *Bbsl*-infected nymphs provided by the Tsao laboratory at Michigan State University. These nymphs been experimentally infected with *Bbss*. *Bbss* infection prevalence of these laboratory-colony nymphs was expected to be ~70%; a subset of the nymphs was tested to estimate the actual prevalence.

I applied 10-12 laboratory-infected nymphs to each skink and allowed them to engorge and detach, after which they were collected into 70% EtOH for testing to determine any change in infection prevalence arising from having fed on the skink. Assuming 70% prevalence, feeding by 10 nymphs represented a >99% chance of each skink being exposed to *Bbss*. Engorged nymphs were tested. I applied ~30 xenodiagnostic larvae at Week 1 post-challenge. These ticks were allowed to feed until they detached and were then collected into 70% EtOH. A second round of 30 xenodiagnostic larvae was applied at Week 6 post-challenge. These ticks were allowed to feed until detached and were collected into two groups. Group 1 was placed into 70% EtOH for immediate testing. Group 2 was maintained alive in a humidity chamber, for use in future transmission experiments if infection was found in the skinks. Week 6

Group 1 larvae were pooled for DNA extraction and real-time PCR testing (two pools per host, with no more than 5 ticks per pool). Week 6 larvae that molted into nymphs (Group 2) were then preserved in 70% EtOH for DNA extraction and real-time PCR testing (two nymphs per extraction, with up to 5 extractions per host).

Five ICR-strain mice were included in Trial 2 as positive experimental controls; these were challenged in the same way as the skinks. Engorged nymphs collected post-feeding on mice, and xenodiagnostic larvae collected and pooled after weeks 1 and 6, were tested for the presence of *Borrelia* spp. using the DNA extraction protocol and real-time PCR procedures described above (Courtney, 2004).

3.3 RESULTS

Infection Trial 1: Naturally infected nymphs

A subsample of the wild nymphs from LD endemic areas – collected for use in the first infestation experiment – was 18.9% infected (7 of 37 nymphs tested positive by qPCR) with *Bbss*. After feeding, engorged nymphs were only 6.9% infected; whereas nymphs that fed on mice were 16.7% infected (Table 3.1).

Xenodiagnosis of 10 of the 11 skinks at 3-4 weeks post-infestation indicated that none had acquired detectable *Borrelia* infection (Table 3.2). However, only one of the four mice tested positive by xenodiagnosis.

Infection Trial 2: Laboratory-infected nymphs

A sub-sample of the laboratory-infected nymphs – supplied by Michigan State University for use in the second infestation experiment – was 71.9% infected with *Bbss*

(64 of 89 nymphs tested positive by qPCR). After feeding, the engorged nymphs that fed on skinks were only 7.4% infected; whereas nymphs that fed on mice were 59.3% infected (Table 3.3).

Xenodiagnosis was undertaken on 15 of the 18 infested skinks at 1 week post-infestation (Table 3.4). Skink 37 transmitted *Bbss* to one of three xenodiagnostic larvae tested from that skink (Table 3.4). This infection was apparently transient, as Skink 37 did not infect any engorged Week 6 xenodiagnostic larvae, nor any of the nymphs molting from Week 6 larvae. No other skink transmitted infection to any larvae or nymphs. In contrast, four of the five mice transmitted infection to Week 1 xenodiagnostic larvae, and all five transmitted to engorged Week 6 larvae and to the molted nymphs (Table 3.4).

Table 3.1. Numbers of wild *I. scapularis* nymphs testing *Borrelia*-positive by qPCR, following engorgement on a) skinks and b) mice. These nymphs originated from Lyme disease endemic areas of the northern U.S., and were estimated to have a pre-engorgement *Bbss* prevalence of 18.9%. The engorged nymphs were collected in 70% ethanol and tested individually.

Host ID	Nymphs Applied	No. collected	No. Tested	No. positive	Prevalence
a) Skink host					
1	18	4	4	0	
5	20	1	1	0	
7	17	2	2	1	
8	19	5	5	0	
9	18	0	0	0	
10	19	0	0	0	
11	17	4	4	0	
12	18	4	4	0	
13	17	3	3	1	
14	20	5	5	0	
15	17	1	1	0	
All skinks	200	29	29	2	6.9%
b) Mouse host					
1	10	7	5	1	
2	10	8	5	1	
3	12	9	5	0	
4	10	6	5	1	
5	6	4	4	1	
All mice	48	34	24	4	16.7%

Table 3.2. Numbers of xenodiagnostic *I. scapularis* larval pools testing *Borrelia*-positive by qPCR, following engorgement on skinks and mice during the first infestation trial. Hosts were infested with larvae 3 and 4 weeks after being infested with naturally-infected nymphs from the northern U.S. Skink 1 and Mouse 3 died before they could be tested.

Host ID	Larvae applied at Week 3	Larvae tested[1]	Larval pools positive	Larvae applied at Week 4	Larvae tested[1]	Larval pools positive
a) Skink host						
5	~30	8	0	29	0	0
7	~30	0	0	37	14	0
8	~30	0	0	33	12	0
9	~30	18	0	35	0	0
10	~30	0	0	34	9	0
11	~30	0	0	33	10	0
12	~30	0	0	30	10	0
13	~30	0	0	30	10	0
14	~30	0	0	33	12	0
15	~30	0	0	34	10	0
b) Mouse host						
1	~50	30	3 of 3	-	-	-
2	~50	22	0	-	-	-
4	~50	30	0	-	-	-
5	~50	30	0	-	-	-

[1] Larvae were pooled (N = at least 3 pools per host, no more than 10 larvae per pool)

Table 3.3. Numbers of laboratory-infected *I. scapularis* nymphs testing *Borrelia*-positive by qPCR, following engorgement on a) skinks and b) mice, during the second infestation trial. These nymphs had acquired infection by being fed as larvae on experimentally-infected mice at Michigan State University, and were estimated to have a pre-engorgement *Bbss* prevalence of 71.9% (see text for details). The engorged nymphs were collected in 70% ethanol and tested individually.

Host ID	Nymphs Applied	No. collected	No. tested	No. positive	Prevalence
a) Skink host					
2	10	3	3	0	
3	10	4	4	0	
4	10	3	3	0	
16	10	7	7	1	
18	10	6	6	0	
19	10	6	6	1	
20	10	5	5	0	
29	11	0	0	-	
30	10	3	3	0	
31	11	1	1	0	
32	11	3	3	0	
33	10	3	3	0	
34	10	4	4	0	
35	10	1	1	0	
36	9	5	5	2	
37	10	7	7	1	
38	10	0	0	-	
40	12	6	6	0	
All skinks	184	67	67	5	7.4%
b) Mouse host					
4	10	8	8	4	
5	10	5	5	4	
7	10	6	6	5	
8	10	8	8	3	
9	10	6	6	4	
All mice	40	27	27	16	59.3%

Table 3.4. Numbers of xenodiagnostic *I. scapularis* larvae, and nymphs molting from Week 6 xenodiagnostic larvae, testing *Borrelia*-positive by qPCR, following engorgement on skinks and mice during the second infestation trial. Hosts were infested with larvae 1 and 6 weeks after being infested with laboratory-infected nymphs supplied by Michigan State University. Skink 38 could not be tested because of a limited supply of larvae. Skinks 2 and 31 died before the Week 6 xenodiagnosis.

Host ID	Larvae applied at Week 1	Larvae tested[1]	Larvae positive	Larvae applied at Week 6	Larvae tested[2]	Larval pools positive	Larvae applied at Week 6 molted into nymphs	Nymph pools positive[3]
a) Skink host								
3	34	16	0	41	11	0	13	0
4	35	2	0	33	10	0	19	0
16	31	14	0	38	8	0	21	0
18	38	9	0	41	10	0	13	0
19	29	12	0	35	10	0	11	0
20	25	9	0	31	13	0	15	0
30	30	3	0	31	9	0	13	0
32	35	5	0	40	13	0	24	0
33	32	9	0	32	6	0	8	0
34	27	10	0	31	8	0	9	0
36	28	1	0	37	9	0	3	0
37	32	3	1	39	6	0	9	0
38	32	4	0	-	-	-	-	-
40	31	1	0	44	8	0	9	0
b) Mouse host								
4	30	6	2	~30	3	1 of 2	5	4 of 5
5	30	3	0	~30	4	1 of 2	4	2 of 5
7	30	10	1	~30	10	2 of 2	10	5 of 5
8	30	17	1	~30	5	1 of 2	9	5 of 5
9	27	9	1	~30	5	1 of 2	11	5 of 5

[1] Week 1 engorged larvae were tested individually.
[2] Week 6 larvae were pooled (2 pools per host, <5 larvae per pool).
[3] Molted nymphs were pooled (5 pools per host, 2 nymphs larvae per pool).

3.4 DISCUSSION

My study supports the hypothesis that lizards have a zooprophylactic effect on infected ticks that feed on them, which consequently suppresses sylvatic cycles of *Bbss*. The zooprophylactic effect is demonstrated in the decrease of infection rates in nymphs after feeding on skinks in both infection trials (statistically significant in the second trial). In the trial with the nymphs collected from LD endemic areas, the infection decreased by 64%; whereas it remained relatively similar in the control mice. This result is suggestive, but not conclusive, of zooprophylaxis by these lizard hosts; the change in prevalence of the skink fed-nymphs relative to the unfed nymphs was not statistically significant (Fisher Exact test; $P = 0.13$ as a 1-tailed test). The trial with laboratory-infected nymphs clearly supports this finding. Infection in laboratory-infected nymphs decreased by 90% after feeding on skinks; whereas, infection only decreased 19% in the control mice. This result is highly suggestive of zooprophylaxis by the lizard hosts, because the change in prevalence of the skink fed-nymphs relative to the unfed nymphs was highly statistically significant (Fisher Exact test; $P < 0.001$). In contrast, the prevalence in the mouse-fed nymphs was not significantly lower than that of the unfed nymphs ($P = 0.16$ as a 1-tailed test). It is apparent that there is an unknown mechanism in skinks that has a zoo-prophylactic effect on *B. burgdorferi* (Levine, et al., 1997; Apperson et al., 1993). If northeastern LD-endemic areas expand, *Bbss*-infected *I. scapularis* populations may move southward. My findings suggest that a shift by these populations to feeding frequently on skinks would help suppress the LD cycle. The question of whether northern

I. scapularis will indeed feed on southern skinks in preference to mice is a question currently under investigation by our research group.

My first experimental infestation, involving nymphs collected from LD endemic areas, were not as effective in transmitting *Bbsl* as I had anticipated. These nymphs, although 18.9% infected, only infected one of the four mice on which they were placed. Five nymphs were placed on each animal, so each mouse had ~65% chance of becoming infected. It is unclear, therefore, why the success in infecting the mice was so low, and this outcome makes it problematic to determine why no skink infection was observed in the first infestation experiment. Nevertheless, even when much more highly-infected nymphs were applied to skinks, in the second trial, persistent infection still did not occur.

Week 1 larva that fed on Skink 37 became infected, but it was not capable of maintaining detectable bacterial infection six weeks past the initial challenge; whereas all control laboratory mice remained infected at Week 6. This suggests that even though a small proportion of skinks may become transiently infective, those individuals could infect only larvae feeding within a week or so afterwards, and even then only a very small proportion of those larvae will acquire infection. This confirms that skinks are poor reservoirs for *Bbsl* when compared to *Peromyscus* spp. mice (LoGiudice et al., 2003) which, once infected, can remain infectious for life (Tsao, 2009). In the northeastern US, the phenology of *I. scapularis* is such that larvae typically feed several weeks later than nymphs. If this phenology occurred in the Southeast it would greatly reduce the efficacy of skinks as reservoirs. However, it does appear from recent surveys (Lyme Gradient Project, unpublished data) and from the frequent co-infestation of skinks with both nymphs and larvae observed in this study that it is not uncommon in the Southeast for

larvae to feed either concurrently with nymphs, or within 6 weeks thereafter. Whether this very limited opportunity for transmission is sufficient to play any significant role in maintaining sylvatic cycles of Bbss in the southeast remains uncertain, and would require quantitative modeling to investigate further.

The inability to create persistent infection in skinks in this study differs from a previous study that was able to infect skinks with *Bbss* for several weeks. Three weeks after challenging skinks with infected nymphs, Levin et al. (1996) reported infection in five out of six skinks and transmission of *Bbss* to 23.6% of the xenodiagnostic larvae in Southeastern five-lined skinks for up to 3 weeks after initial challenge. He concluded skinks are capable of transmitting *Bbss* to larvae from a *P. inexpectatus* and skinks could perpetuate the LD cycle (Levin et al., 1996). Levin et al. (1996) used immunofluorescence assay and cultures to test for *Bbss*; whereas, I used molecular testing only. Immunofluorescence assays and cultures test by looking directly at the organisms infecting the sample; whereas, PCR detects the presence of DNA from the organism. PCR is a very sensitive test which can determine the presence of organisms in the past; therefore it can detect a very low amount of bacteria, which is why it was my test of choice. It is possible that my results reflected the low proportion of Southeastern five-lined skinks in our study (5 of 29 skinks) challenged with infected nymphs; however, I have no data to date to suggest that the tick-host interactions in our experiments different in any significant way because I used two additional *Plestiodon* species.

Several methodological issues were encountered during these experiments. Unfed larvae survived approximately six months when kept in the laboratory at 80F and 90% RH. Larvae close to that date were weaker and moved more slowly (pers. obs.). Week 3

larvae in the first trial may not have attached well to skinks because they were approaching this expiration date. In future trials, I recommend storing xenodiagnostic larvae at a lower temperature to prolong their vigor.

One unexamined aspect of my experimental design is whether or not the relatively high temperature of the lizard husbandry room (up to 32C) might have influenced growth of *Bbss* in the ticks or lizards. Since lizards are ectothermic, it can be assumed their internal temperatures matches or nearly matches the ambient temperatures, and temperatures that high may not be optimum for *Bbss* growth *in vivo* (Strle et al., 1996). Nevertheless, *Bbss* is typically cultured *in vitro* at 33C, and the temperatures of the skink tubs did not approach the 40C threshold at which *Bbss* organisms die (Levin, 1996).

The possibility that infected skinks might exhibit a behavioral fever-response - i.e., that they may change their basking behavior so as to raise their internal temperature above normal levels to rid themselves of internal pathogens (Kluger et al., 1975), is intriguing, particularly given *Bbss*' predilection for the superficial tissues that probably reach the highest temperatures when skinks bask. While I do not believe our experimental tubs provided an opportunity for skinks to raise their temperature in this way, it would be an interesting research opportunity to explore husbandry conditions that more closely mimic free-ranging skink habitats.

My research has confirmed that skinks are very important hosts for *I. scapularis* juveniles and thus may be important for suppressing the LD disease cycle in the southeastern US. My findings support the hypothesis that feeding on skinks, rather than small mammals, may indeed be a reason why *I. scapularis* ticks in the Southeast are

rarely infected with *Borrelia* species capable of causing human disease. Skinks are capable of decreasing prevalence of infection in immature *I. scapularis* that feed on them; and are not well- suited for transmission of *Bbss* to new hosts. Therefore, *Plestiodon* spp. skinks may indeed be diluting the amount of *Bbsl* in natural LD cycles. Future studies should aim to determine more precisely the duration of the rare, transient infection of skinks that I observed, with a view to more accurately modeling whether the skinks are contributing to LD cycle in a way that would affect humans directly.

CHAPTER 4 - CONCLUSIONS

Natural prevalence of *Borrelia* spp. in wild *Plestiodon* skinks

Although there are few confirmed cases of Lyme Disease (LD) in the Southeast, the prevalence of human cases of this disease in this region remains controversial (Herman-Deddes, 2011, Diuk-Wasser and Fish 2012). To expand on a recent study that reported high numbers of infected ticks from wild lizards in the Southeast (Clark et al., 2005; CDC, 2012), I collected 40 blue-tailed skinks, comprising *Plestiodon laticeps, P. fasciatus* and *P. inexpectatus*. These are the most common hosts for the *I. scapularis* juveniles in the Southeast (Apperson et al., 1993). There are conflicting reports on *Bbsl* infection in lizards. Clark et al. (2005) found several individuals and species with infections. European lizards which can also be reservoir competent include *Lacerta spp.* (Foldvari et al., 2009; Vaclav et al., 2011) and *Podarcis spp.* (Ragagli et al., 2011). However, it has been proposed that skinks are zooprophylactic for *Bbss* due to the Western Fence lizard's *Bbss* refractoriness and low amounts of LD in the Southeast (Lane et al., 2006; Apperson et al., 1993).

My study objectives were to determine the natural infection prevalence of *Borrelia* in wild caught *Plestiodon* spp. skinks and, if a *Borrelia* sp. was present, determine the persistence of the *Borrelia* sp.

I determined there are cryptic cycles of an undetermined *Borrelia* spp. found in a low proportion of skinks originating from western Florida. Not all *Borrelia* spp. are pathogenic (Stanek et al., 2012), so this may have no human disease implications. My findings do not support Clark et al. (2005) reports of finding as much as 58% of *P.*

laticeps and 65% of *P. fasciatus* to have *Bbsl* infection, nor do they support his conclusion that the Borrelia present in these lizards was human-infectious *Bbss* and *B. andersonii.*

Plestiodon spp. skink capability to obtain and transmit infection

Lyme disease (LD) is more common in the Northeast and Midwest (CDC, 2012). The ecology of *Borrelia* spp. is dictated by its eastern US vector, *I. scapularis*. In the north, *Peromyscus* mice are the most commonly parasitized and most competent reservoir (Stanek et al., 2012). In the south, the tick host shifts to the *Plestiodon* skinks, which are more common, but may not be good reservoirs (Apperson et al., 1993).

My objective was to complete a laboratory experiment that investigated whether naturally-infected and laboratory-infected *I. scapularis* nymphs could pass *Bbss* infection to uninfected *Plestiodon* spp. skinks in such a way that those lizards would then be capable of transmitting that infection on to naïve, xenodiagnostic larvae.

After challenging *Plestiodon* skinks with naturally and laboratory-infected nymphs, I determined skinks in the southeast are not ecologically important reservoirs of the Lyme Disease (LD) pathogen- *B. burgdorferi* sensu stricto (*Bbss*). Although I was not able to create successful infection by the naturally infected nymphs in more than one control mouse, I did not transmit *Bbss* to the skinks either. Using 72% *Bbss* infected laboratory-infected nymphs, I were able to demonstrate skinks are highly zooprophylactic due to a decrease of infection in the laboratory-infected nymphs by 90% (Fisher Exact test; $P < 0.001$). One *Plestiodon inexpectatus* skink transmitted *Bbss* to one larvae feeding at one week post-challenge became infected. However, it was unable to transmit

to another larva at six weeks post-challenge. Levin et al. (1996) was able to create transmission of *Bbss* with *P. inexpectatus* in the laboratory. My inability to reproduce his results may be from the species I used, persistence diminishing after 5 weeks.

4.1 Future Research Directions

The prevalence of non-*Bbss Borrelia* spp. in skinks and the possibility that such bacteria could be acquired and transmitted by human-biting ticks requires further study. Focusing on an area where there are several cases of LD, high numbers of ticks and large populations of *Plestiodon* skinks may yield more of this unknown *Borrelia*, which would be useful for determining pathogen capabilities.

Plestiodon spp. skink zooprophylactic ability should be researched further to determine the mechanism causing this prophylaxis. Future studies could aim to determine the exact time when persistence diminishes. Behavior of infected skinks may also be an area to study because fever response may be decreasing infection as well. It is important to focus on whether the skinks are contributing to LD cycle in a way that would affect humans directly.

LITERATURE CITED

Apperson, C. S., J. F. Levine, T. L. Evans, A. Braswell and J. Heller. 1993. Relative utilization of reptiles and rodents as hosts by immature *Ixodes scapularis* (Acari: Ixodidae) in the coastal plain of North Carolina, USA. *Experimental and Applied Acarology*, 17: 719-731.

Bunikis, J., U. Garpmo, J. Tsao, J. Berglund, D. Fish and A. G. Barbour. 2004. Sequence typing reveals extensive strain diversity of the Lyme borreliosis agents *Borrelia burgdorferi* in North America and *Borrelia afzelii* in Europe. *Microbiology*, 150: 1741-55.

Cabello FC, Godfrey HP, Newman SA. 2007. Hidden in plain sight: *Borrelia burgdorferi* and the extracellular matrix. *Trends in Microbiology*, **15:** 350–54.

Centers for disease control and prevention (CDC). 2011. "Reported Cases of Lyme Disease-United States, 2011" <http://www.cdc.gov/lyme/resources/ReportedCasesofLymeDisease_2011.pdf> Accessed 29 March 2013.

Centers for disease control and prevention (CDC). "Lyme Disease Data." 20 March 2012. <www.cdc.gov/lyme/stats/index.html> Accessed 29 March 2013.

Chowdri, H. R., J. L. Gugliotta, V. P. Berardi, H. K. Goethert, P. J. Molloy, S. L. Sterling and S. R. Telford. 2013. Borrelia miyamotoi Infection Presenting as Human Granulocytic Anaplasmosis: A Case Report. *Annals of Internal Medicine*, 159(1): 21-27.

Clark, K., A. Hendricks, and D. Burge. 2005. Molecular identification and analysis of Borrelia burgdorferi sensu lato in lizards in the Southeastern United States. *Applied and Environmental Microbiology*, 71: 2616-2625.

Conant, R. and J. T. Collins. 1998. Reptiles and Amphibians: Eastern/ Central North America. Peterson Field Guides. *Houghton Mifflin*.

Courtney, J. W., L. M. Kostelnik, N. S. Zeidner, and R. F. Massung. 2004. Multiplex Real-Time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. *Journal of Clinical Microbiology*, 42(7): 3164-8.

Dennis DT, Nekomoto TS, Victor JC, William SP, Piesman J. 1998. Reported distribution of *Ixodes scapularis* and *Ixodes pacificus* ticks (Acari: Ixodidae) in the United States. *Journal of Medical Entomology, 35*:629 38.

Duik-Wasser, M., and D. Fish. 2012. In Response. *The American Journal of Tropical Medical Hygiene,* 86(6): 1086.

Durden, L. A., J. H. Oliver Jr, C. W. Banks, and G. N. Vogel. 2002. Parasitism of the blacklegged tick, *Ixodes scapularis* (Acari: Ixodidae). *Experimental and Applied Acarology*, 26: 257-66.

Foldvari G., K. Rigo, V. Majlathova, I. Majlath, R. Farkas, and B. Pet'ko. 2009. Detection of *Borrelia burgdorferi* sensu lato in Lizards and Their Ticks from Hungary. *Vector-borne and Zoonotic Diseases*, 9(3): 331-336.

Giery, S. T. and R. S. Ostfeld. 2007. The Role of Lizards in the Ecology of Lyme Disease in two Endemic Zones of the Northeastern United States. *Journal of Parasitology*, 93(3): 511-7.

Girard, Y. A., N. Fedorova and R. S. Lane. 2011. Genetic Diversity of Borrelia burgdorferi and Detection of B. bissettii-Like DNA in Serum of North-Coastal California Residents. *Journal of Clinical Microbiology*, 49(3): 945-954.

Herman-Geddes, M. E. 2012. Letter to the Editor: Yale Lyme disease risk maps are not accurate for the South in 2012. *American Journal of Tropical Medical Hygiene,* 86(6): 1085.

Kerr, K.T. 2012. A northern vs. southern United States comparison of host infestation by the Lyme borreliosis vector, Ixodes scapularis. MS Thesis, Department of Biology, Hofstra University, Hempstead NY. 44pp.

Kierans, J. E., H. J. Hutcheson, L. A. Durden, and J. S. H. Klompen. 1996. Ixodes (Ixodes) scapularis (Acari: Ixodidae): redescription of all active stages, distribution, hosts, geographical variation, and medical and veterinary importance. *Journal of Medical Entomology*, 33 (3): 297-318.

Kollars, T., J. Oliver, P. Kollars, and L. Durden. 1999. Seasonal Activity and Host Associations of *Ixodes scapularis* (Acari: Ixodidae) in Southeastern Missouri. *Journal of Medical Entomology*, 36: 720-726.

Kluger, M. J., D. H. Ringler, and M. R. Anver. 1975. Fever and Survival. *Science*, 188(4184): 166-8.

Kuo, M.M., R. S. Lane and P. C. Giclas. 2000. A comparative study of mammalian and reptilian alternative pathway of complement-mediated killing of the Lyme disease spirochete (*Borrelia burgdorferi*). *Journal of Parasitology*, 86(6): 1223-8.

Lane, R., J. Mun, L. Eisen, and R. Eisen. 2006. Refractoriness of the western fence lizard (*Sceloporus occidentalis*) to the Lyme disease group spirochete *Borrelia bissettii*. *Journal of Parasitology*, 92: 691-696.

Levin, M. L., J. E Levine, S. Yang, P. B. Howard, and C. S. Apperson. 1996. Reservoir competence of the southeastern five-lined skink (*Eumeces inexpectatus*) and the

green anole (*Anolis carolinensis*) for *Borrelia burgdorferi*. *American Journal of Tropical Medicine and Hygiene*, 54: 92-97.

Levine, J. F., Apperson, C., P. Howard, M. Washburn, and A. Braswell. 1997. Lizards as Hosts for Immature *Ixodes scapularis* (Acari: Ixodidae) in North Carolina. *Journal of Medical Entomology*, 34: 594-598.

LoGiudice, K., R. S. Ostfeld, K. A. Schmidt and F. Keesing. 2003. The ecology of infectious disease: Effects of host diversity and community composition on Lyme disease risk. *PNAS*, 100(2): 567-571.

Mather, T. N., M. L. Wilson, S. I. Moore, J. M. C. Ribeiro and A. Spielman. 1989. Comparing the Relative Potential of Rodents as Reservoirs of the Lyme Disease Spirochete (*Borrelia burgdorferi*). *American Journal of Epidemiology*, 130 (1): 143-150.

Mulder, P. G., M. K. Harris and R. A. Grantham. 2012. Biology and Management of the Pecan Weevil (Coleoptera: Curculionidae). J. Integrated Pest Management, 3(1).

Ogden, N. and J. Tsao. 2009. Biodiversity and Lyme disease: Dilution or amplification? *Journal of Epidemics*, 1: 196-206.

Ostfeld, R. S. and F. Keesing. 2001. Biodiversity and Disease Risk: the Case of Lyme disease. *Conservation Biology*, 14(3):722-8.

Piesman, J., J. G. Donahue, T. N. Mather, and A. Spielman. 1986. Transovarially acquired Lyme disease spirochetes (*Borrelia burgdorferi*) in field-collected larval *Ixodes dammini* (Acari: Ixodidae). *Journal of Medical Entomology*, 23(2): 219-219(1).

Ragagli, C., L. Bertolotti, M. Giacobini, A. Mannelli, D. Bisanzio, G. Amore, and L. Tomassone. 2011. Transmission Dynamics of *Borrelia lusitaniae* and *Borrelia afzelii* Among *Ixodes ricinus*, Lizards, and Mice in Tuscany, Central Italy. *Vector-Borne and Zoonotic Diseases*, 11(1): 21-28.

Rosen, M. E. 2009. Investigating the maintenance of the Lyme disease pathogen, *Borrelia burgdorferi*, and its vector, *Ixodes scapularis*, in Tennessee. Thesis for Master of Science Degree for the University of Tennessee, Knoxville.

Rudenko, N., M. Golovchenko, T. Lin, L. Gao, L. Grubhoffer, and J. H. Oliver, Jr. 2009. Delineation of a New Species of the *Borrelia burgdorferi* Sensu Lato Complex, *Borrelia americana* sp. nov. *Journal of Clinical Microbiology*, 47 (12): 3875-80.

Stafford, K. C. 1994. Survival of Immature *Ixodes scapularis* (Acari: Ixodidae) at Different Relative Humidities. *Journal of Medical Entomology*, 31(2): 310-314.

Stanek, G., G. P. Wormser, J. Gray, and F. Strle. 2012. Lyme borreliosis. *Lancet*, 379:461-73.

Strle, F., J. A. Nelson, E. Ruzic-Sabljic, J. Cimperman, V. Maraspin, S. Lotric-Furlan, Y. Cheng, M. M. Picken, G. M. Trenholme, R. N. Picken. 1996. European Lyme Borreliosis: 231 Culture-Confirmed Cases Involving Patients with Erythema Migrans. *Clinical Infectious Diseases*, 23: 61-5.

Stromdahl, E. Y. and G. J. Hickling. 2012. Aetiology of Tick-borne Human Diseases with Emphasis on the South-Eastern United States. *Zoonoses and Public Health*, 59(2): 48-64.

Swanson, K., and D. Norris. 2007. Detection of *Borrelia burgdorferi* DNA in lizards from Southern Maryland. *Vector Borne and Zoonotic diseases*, 7: 42-49.

Vaclav, R., M. Ficová and P. Prokop. 2011. Associations Between Coinfection Prevalence of *Borrelia lusitaniae*, *Anaplasma* sp., and *Rickettsia* sp. in Hard Ticks Feeding on Reptile Hosts. *Microbial Ecology*, 61: 245-253.

APPENDICES

Appendix 1: DNA extraction

1st Day Protocol

*Using Qiagen DNeasy Blood and Tissue DNA Extraction Kit

1. Prepare the number of clean 1.5 mL microcentrifuge vials needed for extraction. Label with trial and unique identifier.
2. Remove sample from ethanol and allow to dry on paper towel or weigh boat.
3. Place ticks in respective labeled vials from step 1.
4. Add 180 µL of ATL lysis buffer to each vial.
5. Within each vial, macerate each tick using an individual micropestle for each sample. Make sure to expose the midgut of the tick.
    - Can submerge vial into liquid nitrogen to make the chitin exterior more brittle and easier to macerate.
6. Add 40 µL of Proteinase-K to each sample.
7. Vortex each sample thoroughly and place in a shaking machine at 56C for at least 12 hours.

2nd Day Protocol

1. Set a heating block to 72C.
2. Prepare and label vials for each tick sample: 1 set of column vials from kit and 2 sets of 1.5 mL microcentrifuge vials.
    - Label with unique identifier, trial, and elution number (2 elutions per sample).

3. Add 220 µL of AL buffer to each sample. Vortex well. Place in pre-heated block (72C) for 10 minutes.
4. Add 250 µL of 100% ethanol to each sample. Vortex.
5. Transfer all liquid contents into a column vial (~700 µL). Be careful to avoid body parts. Centrifuge column vials for 1 minute at 12,000 rpm. Place column vial in a new collection tube.
    - Store left-over body parts at -20C.
6. Add 500 µL AW1 buffer. Centrifuge column vials for 1 minute at 12,000 rpm. Place column vial in a new collection tube.
7. Add 500 µL AW2 buffer. Centrifuge column vials for 1 minute at 12,000 rpm. Place column vial in a new collection tube.
8. Spin again for 2 minutes at 12,000 rpm. Place column vial in pre-labeled 1.5 mL microcentrifuge tubes for elution 1.
9. Add 50 µL hot molecular grade water into column vial. Place directly on filter. Let sit for ~5 minutes. Centrifuge for 1 minute at 12,000 rpm.
10. Repeat steps 8 & 9 for elution 2.

APPENDIX 2: REAL-TIME PCR TESTING

Primer sequences:

- Bb23Sf: 5`-CGAGTCTTAAAAGGGCGATTTAGT
- Bb23Sr: 5`-GCTTCAGCCTGGCCATAAATAG
    - Labeled at the 5` and 3` ends with 6-carboxy-fluorescein (6-FAM)

Probe sequence:

- Bb23Sp-FAM: 5`-AGATGTGGTAGACCCGAAGCCGAGTG
    - Labeled at the 5` and 3` ends with 6-carboxyl-tetramethyl-rhodamine (TAMRA)

1. Prepare a tabled sheet for your test indicating which sample will go in each well.
    - Make sure to allow for a negative control (water) in the first well and between each group of samples. Positive controls go in the last wells. Include a tick positive as well as the laboratory positive.
    - Organize tube rack to mimic the prepared sheet.
2. Each reaction will have the following master mix:
    - 0.4 µL Roxidine II dye
    - 1 µL Assci primer/probe
    - 6.6 µL Molecular grade water
    - 10 µL Taqman (keep on ice)
3. Make enough master mix for each of your samples.
4. Place 18 µL of master mix in each sample well on the 48-well plate.
5. Place 2 µL DNA or control sample in each corresponding well.
6. Seal the plate tightly. Vortex. Centrifuge plate for 2 minutes at 12,000 rpm.

7. Using Real-time PCR machine, assign the plate to test for “Lyme.” Assign each well with the respective samples.

APPENDIX 3: NESTED PCR PROTOCOL BY CATHY SCOTT

Outer IGS PCR 1:
IGS F: 5' – GTA TGT TTA GTG AGG GGG GTG – 3' = 21 bases
IGS R: 5' – GGA TCA TAG CTC AGG TGG TTA G – 3' = 22 bases

Amplification conditions:

Denaturation	3 min @ 95C	
Denaturation	30 sec @ 95C	5 touchdown cycles decreasing temperature -2/cycle
Annealing	30 sec @ 65C	
Elongation	60 sec @ 72C	
Denaturation	20 sec @ 88C	25 cycles
Annealing	40 sec @ 56C	
Elongation	60 sec @ 72C	
Final elongation	7 min @ 72C	

1. PCRbeads: Do not mix the tube contents until all the components (below) have been added to the tube containing the bead.
2. Make a master mix for each sample:

   IGS F: primer (final concentration 1µM) 5µl

   IGS R: primer (final concentration 1µM) 5µl

   Sterile high-quality water to a final volume of 11 µl
3. Add 21 µl of Master mix to each PCR bead tube
4. Add Tick DNA 4µl

5. Snap the caps (provided) onto the tubes, pushing down firmly to ensure a tight fit. Mix the tube contents by gently flicking the tube with a finger.
6. Place the reaction mixtures on ice or in a cold block until ready for cycling. Minimize the time on ice prior to cycling to prevent formation of background reaction products.

Nested IGS PCR 2:

Repeat steps 1-6 using the nested primer set and 4µl of the outer PCR reaction.

IGS Fn: 5' – AGG GGG GTG AAG TCG TAA CAA G – 3' = 22 bases
IGS Rn: 5' – GTC TGA TAA ACC TGA GGT CGG A – 3' = 22 bases

Amplification conditions:

Denaturation 3 min @ 95C

Denaturation 30 sec @ 95C
Annealing 30 sec @ 65C } 5 touchdown cycles decreasing temperature -1/cycle
Elongation 60 sec @ 72C

Denaturation 20 sec @ 88C
Annealing 40 sec @ 60C } 25 cycles
Elongation 60 sec @ 72C

Final elongation 7 min @ 72C

Primer Sequences- Stock concentration 1mM- working concentration 5µM- Sequencing concentration 10 µM

VITA

Teresa Moody is originally from Ardmore in Giles County; TN. Her passion for animals inspired her to work at a veterinary clinic and pursue agricultural classes while in high school. She attended the University of Tennessee at Martin for her Bachelor of Science degree in Natural Resource Management concentrating on Wildlife Biology and graduated in May 2011. She has worked various seasonal jobs for the US Fish and Wildlife Service, Tennessee Wildlife Federation with Great Outdoors University, Illinois Natural History Survey and Tennessee Wildlife Resources Agency. Teresa first worked on the Lyme Gradient Project in May 2011 as a research technician. In January 2012 she enrolled in the Masters of Science Program at the University of Tennessee Knoxville, where her thesis research focused on the role of Plestiodon skinks as potential reservoirs for the Lyme disease agent *B. burgdorferi*. She has been an active member of The Wildlife Society since 2007. Teresa received multiple awards for presentations on her research during her Masters studies.